# Supplementary Information

## Selective inhibition of HDAC6 promotes bladder cancer radiosensitization and mitigates the radiation-induced CXCL1 signalling

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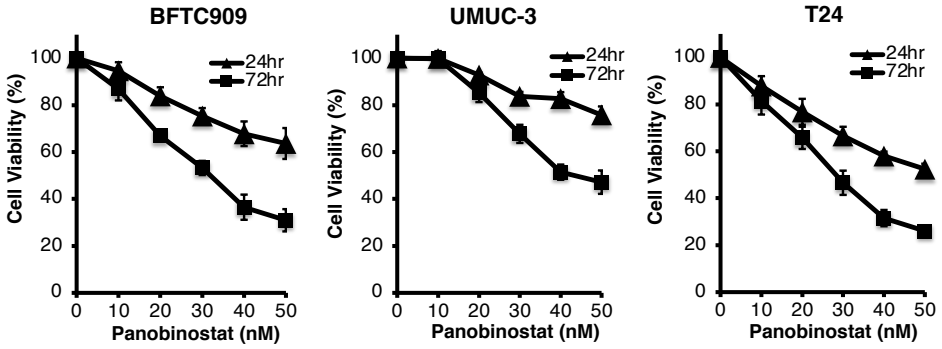


**Figure S1. The viability of BFTC909, UMUC-3 and T24 cells treated with panobinostat (nM).** The half maximal inhibitory concentration ($IC_{50}$) of panobinostat in different bladder cancer cell lines were analyzed at 24 and 72 hours by the CCK-8 assay. Data are presented as the means ± SD from three independent biological replicates.

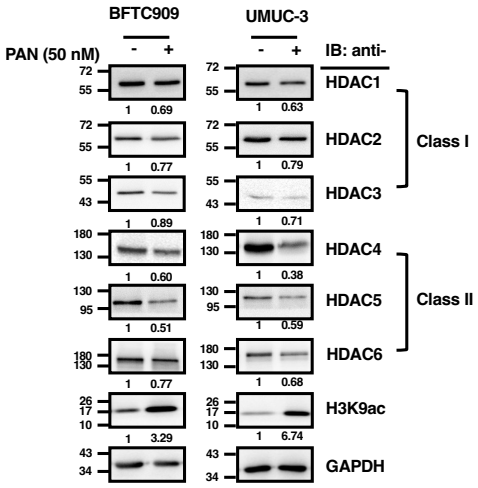


**Figure S2. Protein expression of HDACs treated with panobinostat in bladder cancer cell lines.** Western blotting analysis for class I (HDAC1, HDAC2, HDAC3) and class II (HDAC4, HDAC5, HDAC6) HDACs, and acetyl-histone H3 (Lys9) (H3K9ac) in BFTC909 and UMUC-3 cells with or without 50 nM panobinostat (PAN) treatment for 16 hours. GAPDH was used as the internal control.

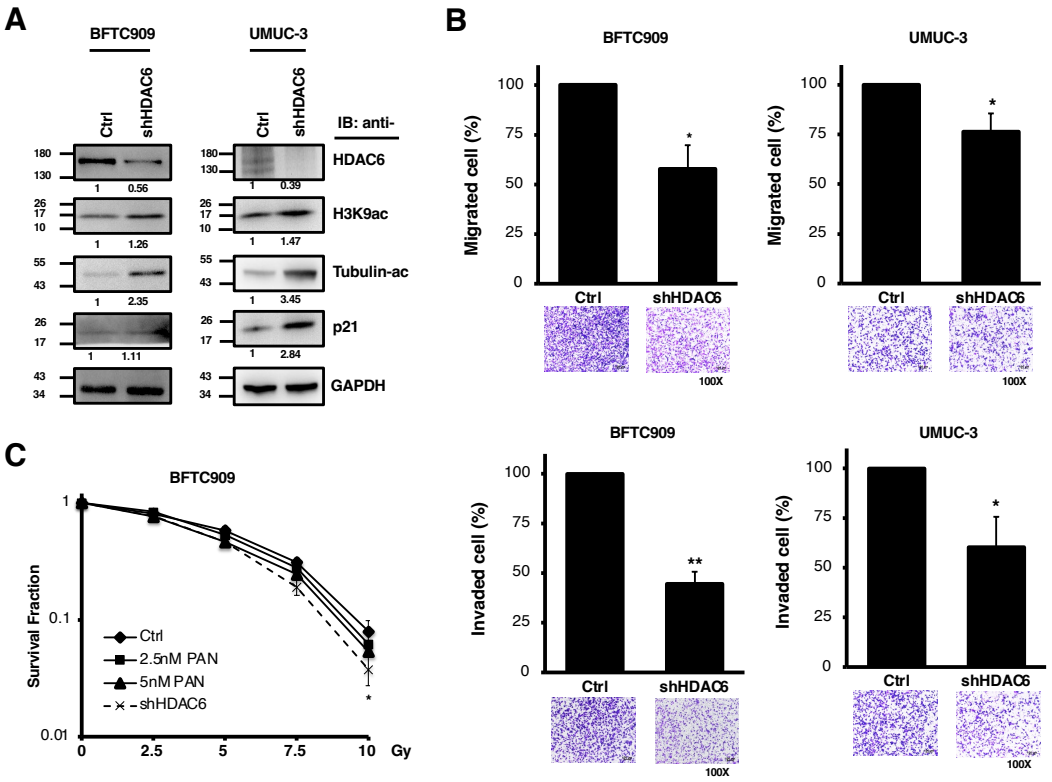


**Figure S3**. **HDAC6 knockdown resulted in decreased migration and invasion abilities of BFTC909 and UMUC-3 cells. a** Western blots showing the levels of HDAC6, H3K9ac, acetyl-α-tubulin and p21 in control and shHDAC6 BFTC909 cells. GAPDH was used as the internal control. **b** Cell migration and invasion abilities were examined based on the numbers of stained migrated (16 hours) and invaded (20 hours) cells on the lower surface of the Transwell membrane viewed under a microscope. Representative images of the control and shHDAC6-transfected cells (left panel: BFTC909 cells; right panel: UMUC-3 cells) are shown below. **c** Clonogenic survival of control and shHDAC6 BFTC909 cells. Colonies were stained with 0.25% crystal violet at day 7, and the survival rate was calculated. Data are presented as the means ± SD from three independent biological replicates. Student's t test was used to determine significant differences. * $P < 0.05$; ** $P < 0.01$.

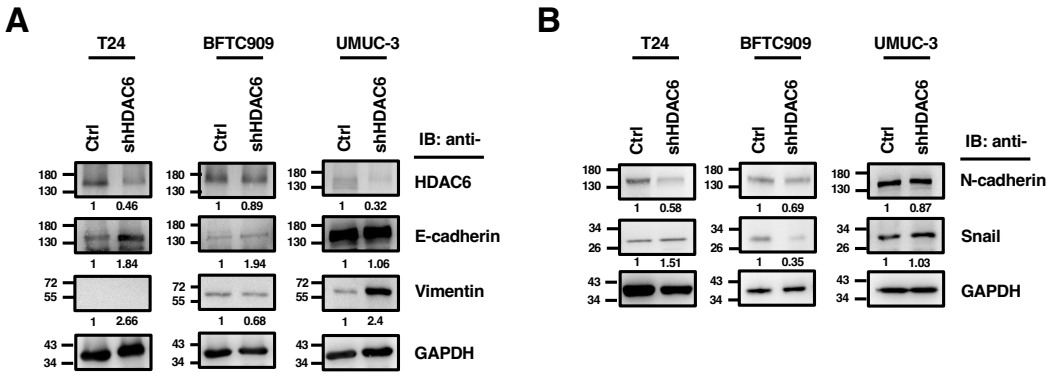


**Figure S4. Protein expression of EMT markers in HDAC6 knockdown bladder cancer cell lines.** Western blot analysis of E-cadherin, Vimentin, N-cadherin and Snail levels in T24, BFTC909 and UMUC-3 cells with (shHDAC6) or without (Ctrl) HDAC6 knockdown. GAPDH was used as the internal control.

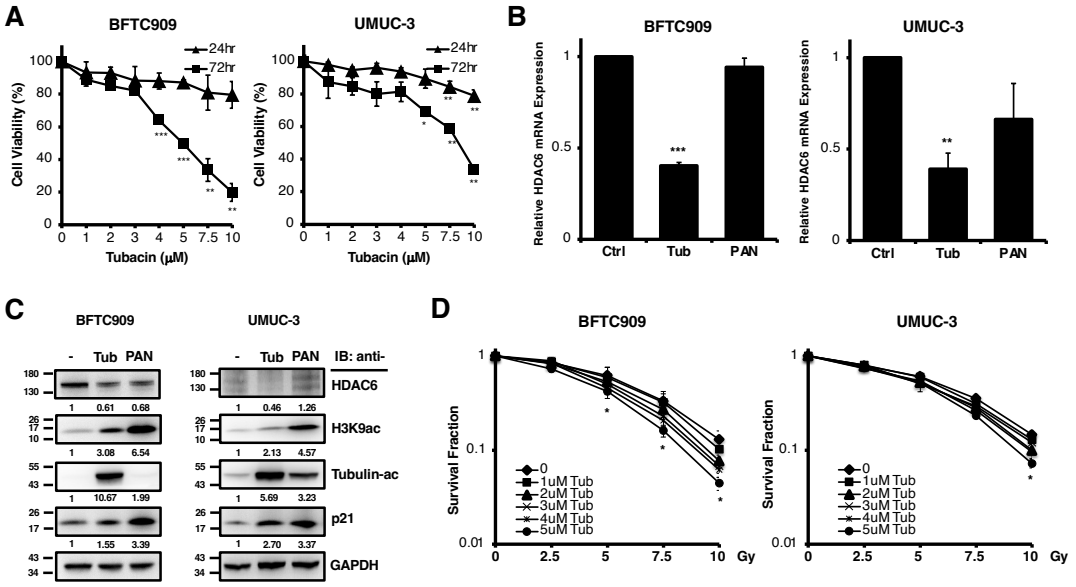


**Figure S5. The radiosensitizing effect of the HDAC6-specific inhibitor tubacin on BFTC909 and UMUC-3 cells. a** The viability of BFTC909 and UMUC-3 cells treated with different concentrations of tubacin (μM) was analyzed at 24 and 72 hours using the CCK-8 assay. **b** HDAC6 mRNA expression in BFTC909 and UMUC-3 cells treated with tubacin (Tub) was assessed by qPCR. Panobinostat (PAN) treatment was used for comparison. **c** Western blots showing the levels of HDAC6, H3K9ac, acetyl-α-tubulin and p21 in BFTC909 and UMUC-3 cells treated with tubacin (Tub) or panobinostat (PAN) for 16 hours. GAPDH was used as the internal control. **d** Clonogenic survival of BFTC909 and UMUC-3 cells treated with tubacin (Tub) followed by irradiation (Gy). Colonies were stained with 0.25% crystal violet on day 7, and the survival rate was calculated. Data are presented as the means ± SD from three independent biological replicates. Student's t test was used to determine significant differences. * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$, compared to the control.

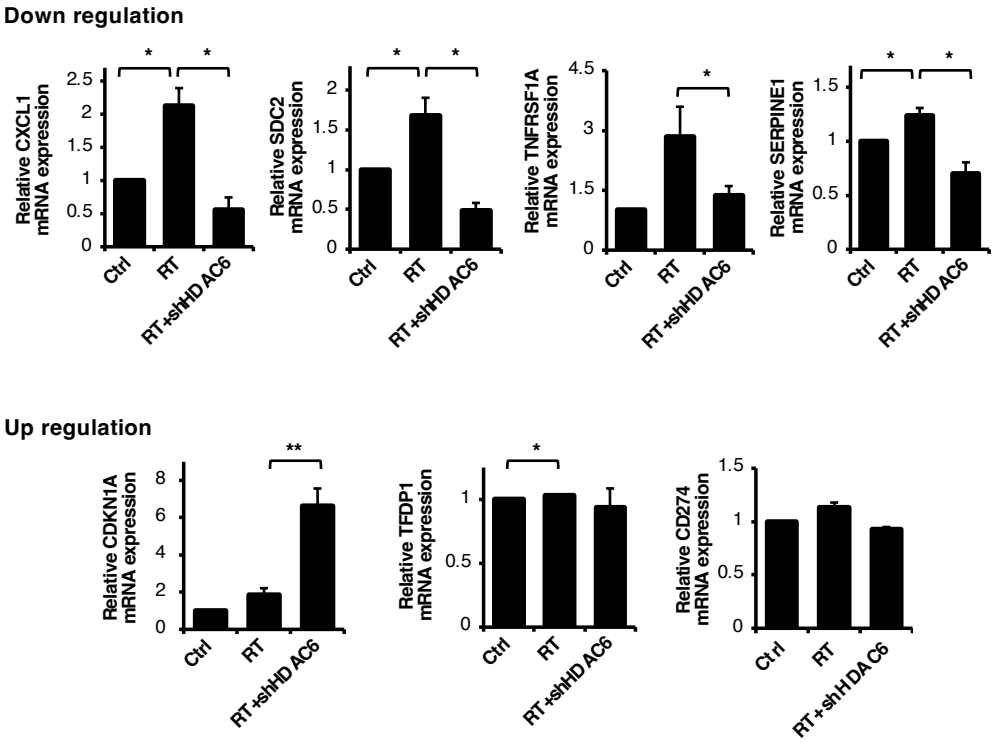


**Figure S6. Validation of differentially expressed genes (DEGs) up- and downregulated, respectively, in RT+shHDAC6 cells compared to RT alone cells.** mRNA levels of DEGs were analyzed by qPCR in control (Ctrl) T24 cells treated with or without 5 Gy irradiation (RT), and shHDAC6 T24 cells treated with RT (RT+shHDAC6). Data are presented as the means ± SD from three independent biological replicates. Student's *t* test was used for significant differences. * $P < 0.05$; ** $P < 0.01$.

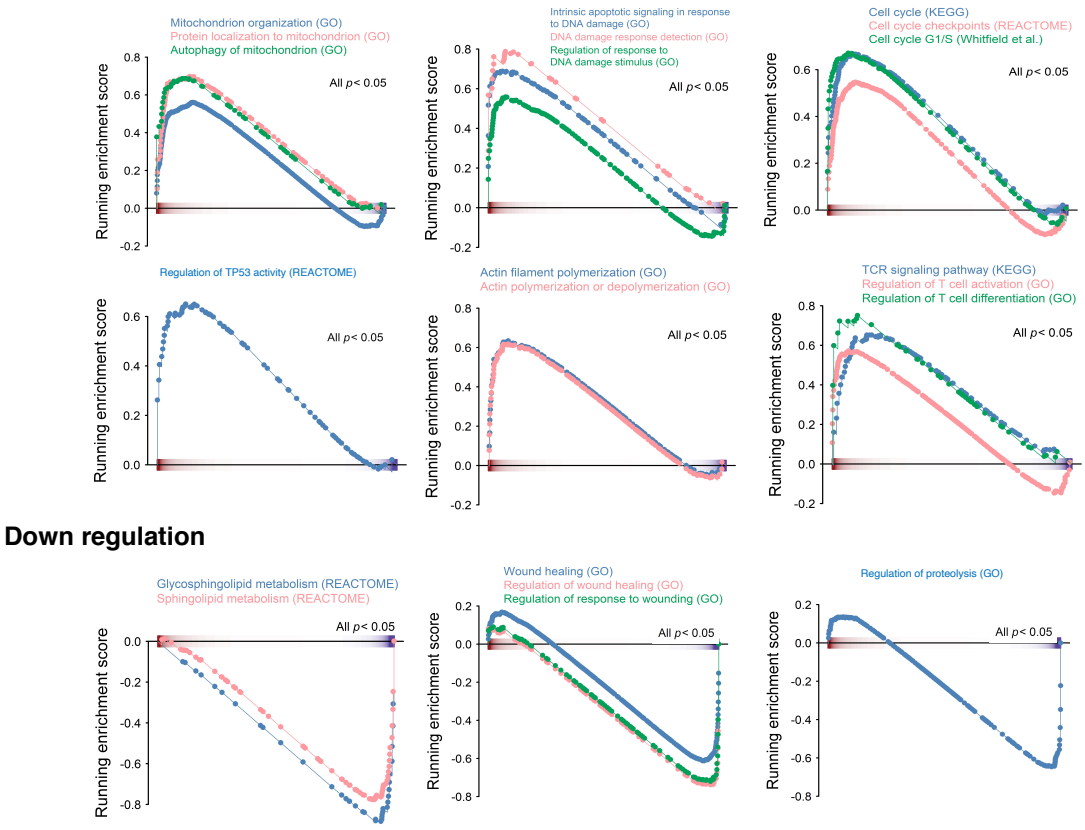


**Figure S7. Gene set enrichment analysis (GSEA) of the DEGs in irradiated T24 with HDAC6 knockdown.** GSEA enrichment plots for pathways of mitochondrion organization, DNA damage response, cell cycle checkpoint, signalling by TP53, actin filament polymerization, T cell activation; and membrane lipid metabolism, wound healing and proteolysis in RT+shHDAC6 T24 cells compared to RT alone cells.

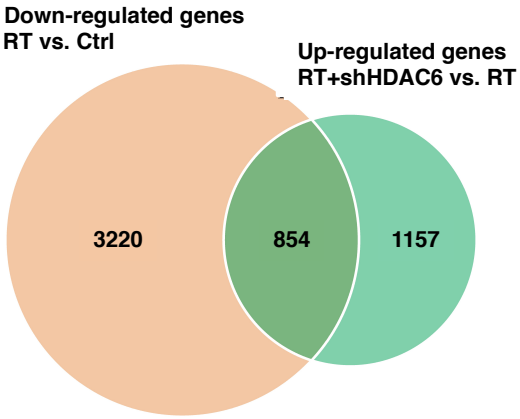


**Figure S8. Numbers of irradiation-downregulated genes that were increased by HDAC6 knockdown in T24 cells.** The Venn diagram shows the overlap between the gene set of downregulated genes in T24 cells treated with RT compared to untreated cells (Ctrl) and the gene set of upregulated genes in RT+shHDAC6 T24 cells compared to RT alone cells.

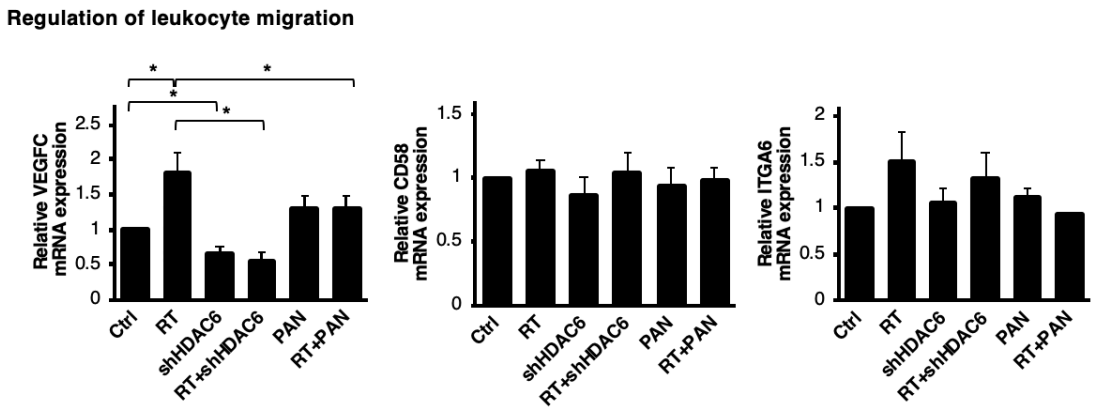


**Figure S9. Validation of differentially expressed genes (DEGs) in RT+shHDAC6 cells compared to RT alone cells.** mRNA levels of VEGFC, CD58 and ITGA6 were analyzed by qPCR in control and shHDAC6 T24 cells treated with or without 5 Gy irradiation (RT). 10 nM panobinostat (PAN) was used for comparison. Data are presented as the means ± SD from three independent biological replicates. Student's t test was used for significant differences. * $P < 0.05$.

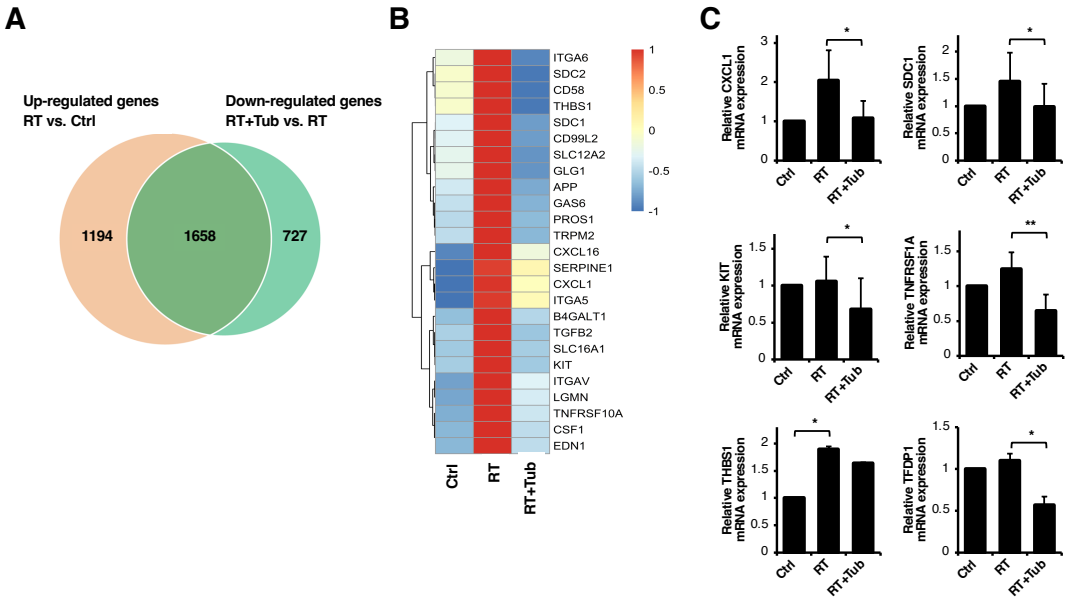


**Figure S10. RNA-seq analysis of T24 cells treated with tubacin, an HDAC6 inhibitor. a** The Venn diagram shows the overlap between the gene set of upregulated genes in T24 cells treated with RT compared to untreated cells (Ctrl) and the gene set of downregulated genes in T24 cells treated with radiation and tubacin (RT+Tub) compared to that treated with RT alone. **b** Heatmap of gene expression from the overlapping gene set shown in **a** in the control, RT and RT+Tub T24 cells. **c** q-PCR validation of DEG genes in control (Ctrl) T24 cells treated with or without 5 Gy irradiation (RT), and T24 cells treated with tubacin and RT (RT+Tub). Data are presented as the means ± SD from three independent biological replicates. Student's *t* test was used for significant differences. * $P < 0.05$; ** $P < 0.01$.

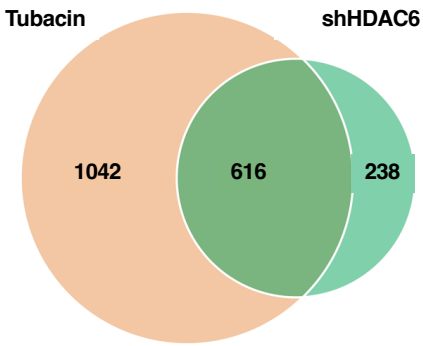


**Figure S11. The effect of tubacin and HDAC6 knockdown on the radiation-induced upregulated genes**. The Venn diagram shows the overlap between the gene set of RT-induced genes suppressed by tubacin and the gene set of RT-induced genes suppressed by the knockdown of HDAC6.

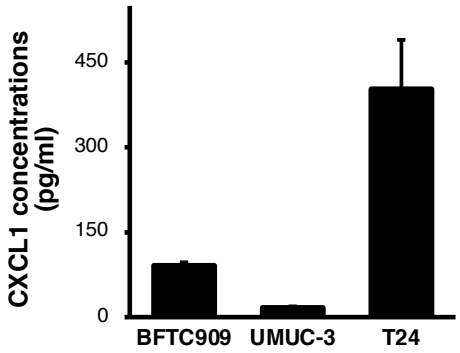


**Figure S12. CXCL1 protein expression in human bladder cancer cell lines**. CXCL1 protein levels in conditioned medium from BFTC909, UMUC-3 and T24 cells were measured by ELISA. Data are presented as the means ± SD from three independent biological replicates.

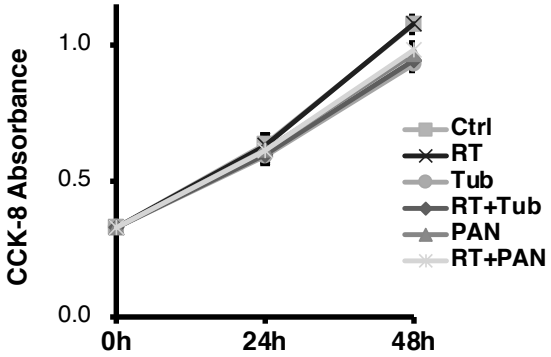


**Figure S13. Quantification of viable cells under radiation, tubacin, panobinostat and combination treatment.** Growth curves of T24 cells with or without 5 μM **tubacin** (Tub) treatment followed by 5 Gy irradiation (RT) were determined by the CCK-8 assay at 24 and 48 hours. Panobinostat (PAN, 10 nM) was used for comparison. Data are presented as the means ± SD from three independent biological replicates.

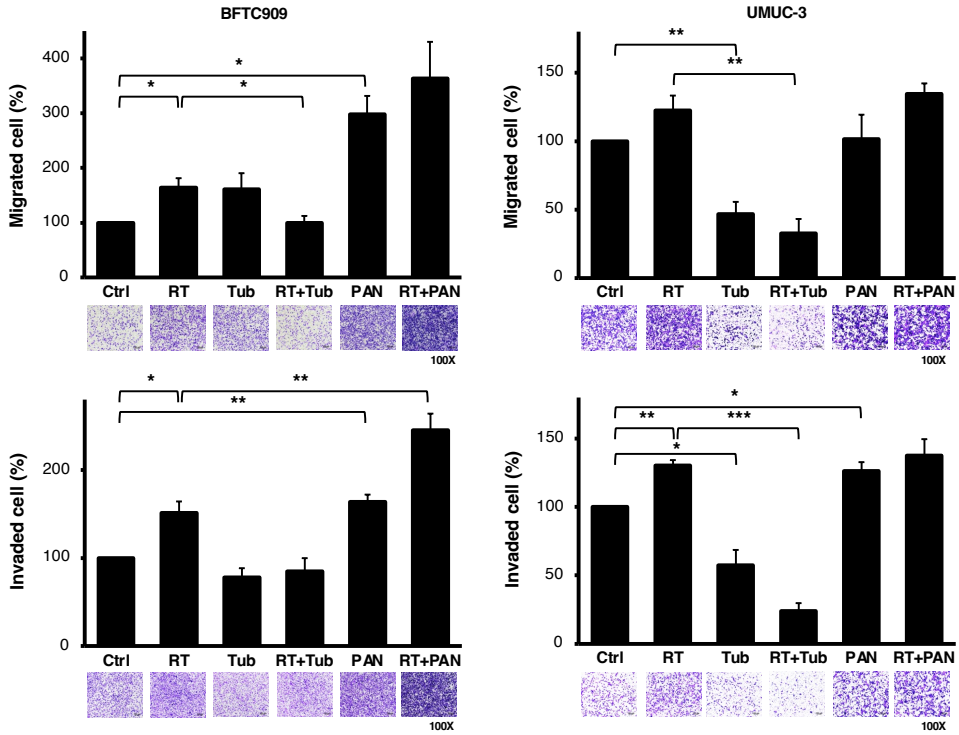


**Figure S14. Tubacin combined with RT repressed the RT-induced migration/invasion of BFTC909 and UMUC-3 cells.** Migration and invasion assays of BFTC909 and UMUC-3 cells treated with or without tubacin (Tub) followed by 5 Gy irradiation (RT). Migrated and invaded cells on the lower surface of the Transwell membrane were stained and counted at 16 and 20 hours, respectively. Representative images are shown below individually. Panobinostat (PAN) was used for comparison. Data are presented as the means ± SD from three independent biological replicates. Student's t test was used to determine significant differences. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

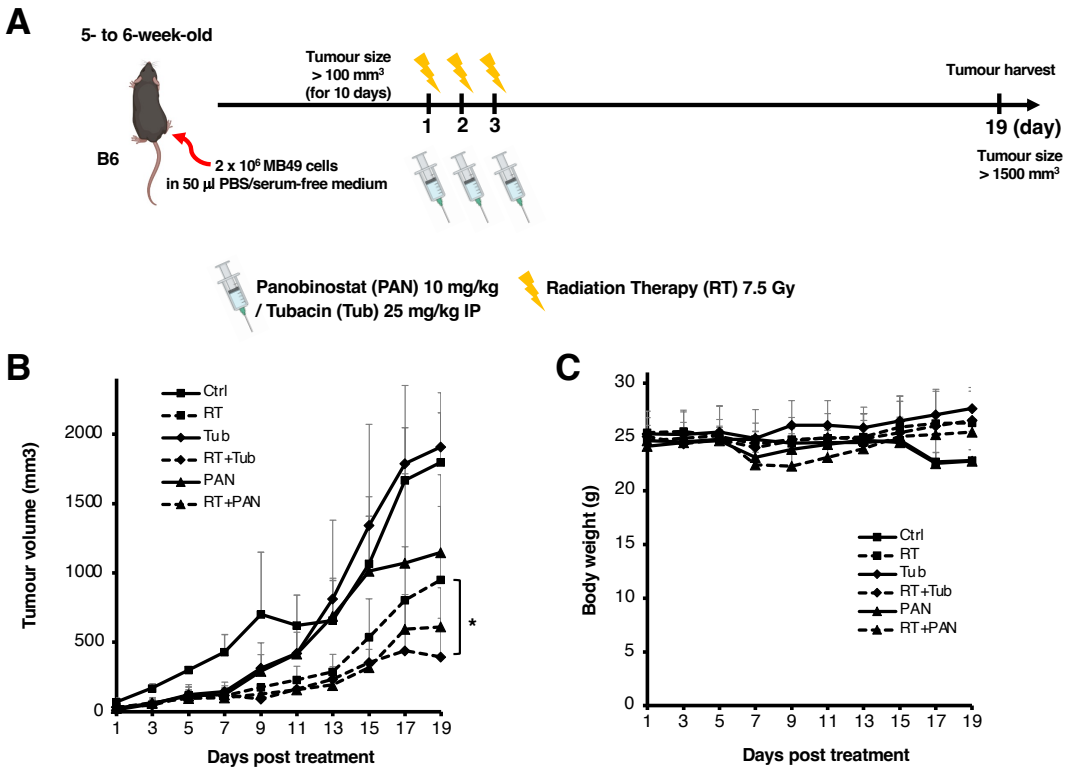


**Figure S15. The HDAC6i tubacin exerts a radiosensitizing effect on tumor growth in vivo.** **a** Timeline of the analysis of the ectopic tumor model. MB49 cells were injected subcutaneously into the right hind limb of thirty C57BL/6 mice. The experimental group was unexposed or exposed to 7.5 Gy of RT, and tubacin or panobinostat was administered by intraperitoneal injection for three days. Mice were sacrificed on day 19. **b** Tumor volumes were measured for each desired treatment group on the indicated days (n=5 for each group). **c** The body weight of each mouse group was recorded after the desired treatments on the indicated days (n=5 for each group). Data are presented as the means ± SD for each independent biological replicate. Student's t test was used to determine significant differences. * $P < 0.05$.

**Table S1. List of antibodies, related to experimental procedures**

| Used for | Official Name | Cat. # | Distributor |
|---|---|---|---|
| WB | Acetyl-α-Tubulin (Lys40) | #5335 | Cell Signaling |
| | Acetyl-Histone H3 (Lys9) | #9649 | Cell Signaling |
| | Akt | #9272 | Cell Signaling |
| | E-Cadherin | #14472 | Cell Signaling |
| | GAPDH | #2118 | Cell Signaling |
| | HDAC1 | #5356 | Cell Signaling |
| | HDAC2 | #5113 | Cell Signaling |
| | HDAC3 | #3949 | Cell Signaling |
| | HDAC4 | #7628 | Cell Signaling |
| | HDAC5 | #20458 | Cell Signaling |
| | HDAC6 | #7558 | Cell Signaling |
| | N-Cadherin | #13116 | Cell Signaling |
| | p21 Waf1/Cip1 | #2946 | Cell Signaling |
| | Phospho-Akt (Ser473) | #9271 | Cell Signaling |
| | Phospho-PTEN (Ser380/Thr382/383) | #9549 | Cell Signaling |
| | PTEN | #9188 | Cell Signaling |
| | Rad51 | #8875 | Cell Signaling |
| | Snail | #3879 | Cell Signaling |
| | Vimentin | #5741 | Cell Signaling |
| **IF** | Phospho-Histone H2A.X (Ser139) | #9718 | Cell Signaling |
| **IHC** | GROα | sc-514065 | Santa Cruz |
| **Neutralized** | Human/Primate CXCL1/GROα/KC/CINC-1 | MAB275 | R&D Systems |

**Table S2. List of primers**

| Gene Symbol | Forward 5'-3' | Reverse 5'-3' |
|---|---|---|
| CD58 | TGT GCT TGA GTC TCT TCC ATC | ATT GCT CCA TAG GAC AAT CCC |
| CD274 | CCT CAA GTG TCT GTG CAG TAT C | TAT CAC AAC AGG GTG GTT ACA G |
| CDKN1A | CGC TCT ACA TCT TCT GCC TTA G | CGG GAT GAG GAG GCT TTA AAT A |
| CXCL1 | CAC AGT GTG TGG TCA ACA TTT C | AGT AAA GGT AGC CCT TGT TTC C |
| CXCL16 | CAT CTT CAT CCT CAC CGC AG | CCT ATA ATC CTC GCA CCT TCA G |
| EDN1 | TGG TAA CTG CTT TGG TCT CTT C | TTC TCC CTG AAA TGT GCC AG |
| GAPDH | CCA CTC CTC CAC CTT TGA C | ACC CTG TTG CTG TAG CCA |
| HDAC6 | GTC TAC TGT GGT CGT TAC ATC | GGC CTG ACA GTA GTA ACA C |
| ITGA6 | CAC AGG TTC TCA AGG GTA TAT CAC | AAC AGC AAC ATC AGG GTA GG |
| KIT | ACA TAG ACC CAA CAC AAC TTC C | GCT TCC CGT TCT GTC AAA TG |
| KRT7 | GTG GTG GAG GAC TTC AAG AAT AA | CAT TTG GCC ATC TCC TCA TAC T |
| SDC1 | CCC CAT CTT GCT TCC CTA ATC | CAG ATA GTC CAT ACC CTG TTG C |
| SDC2 | GCC TGT CGA TGA GAC TGT TTA T | GGC AGC CTT CCA ATA TCT CTA C |
| SERPINE1 | GCC CTT GAG TGC TTG TTA GA | GGT GAC ACA GAC CAC AAA GA |
| TFDP1 | GCT CTG CCG AAG ACC TTA AA | GAG AAC CTT GTG CCG TTA GA |
| TGFB2 | CTA CCC TAA GCG AGC AAT TCC | TCC AAA CGC CAA CCC AG |
| THBS1 | GAG ACG GTA TCC TCA ATG AAC G | TCT CCC TTG CCA TCT TTG TC |
| TNFRSF1A | GAA GAA CCA GTA CCG GCA TTA | TCC TCA GTG CCC TTA ACA TTC |
| VEGFC | GAC CCC ACA AAG AAC TAG ACA G | CAT TCA CAG GCA CAT TTT CCA G |